Epidemiol. Infect. (1992), **109**, 121–131
Printed in Great Britain

The ability of airborne *Klebsiella pneumoniae* to colonize mouse lungs

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(*Accepted 14 February 1992*)

SUMMARY

A strain of *Klebsiella pneumoniae* was aerosolized and its survival in air at different relative humidities was studied. Survival was dependent upon relative humidity and aerosols were most stable during storage at a relative humidity of 60%. Mice were exposed to aerosols of *K. pneumoniae* produced at this humidity and lung samples taken at timed intervals after exposure. Fifteen strains of *K. pneumoniae* were tested for their ability to colonize mice, but only five were detectable in mouse lungs 7 days after exposure. Three of these strains persisted without an increase in bacterial numbers, regardless of the initial inoculum used. Two strains of *K. pneumoniae*, designated strains 15 and 16, persisted in a similar manner when used at a low dose; however, when the dose received per lung was increased there was a rapid multiplication of bacteria in the lungs.

INTRODUCTION

Pathogenic bacteria capable of causing respiratory disease can be transmitted by the aerosol route. Natural processes such as breathing and sneezing produce aerosols [1], thus an animal harbouring respiratory bacteria will produce airborne bacteria. If these are inhaled by a susceptible host, the bacteria may cause disease. Large aerosol droplets are deposited fairly quickly after generation, but particles of 1–5 μm diameter can remain airborne for several hours [2]. There is therefore a potential for respiratory pathogens to be present as aerosols out of the immediate vicinity of a diseased host.

In order for airborne bacteria to cause disease they must survive the aerosolization process and remain infective whilst existing in an aerosol. Gram-negative bacteria are particularly susceptible to damage during aerosolization; this may occur at the outer membrane [3] in addition to other sites. The damage may be so severe that there is a loss in viability of the bacteria. Thus the capability of an inhaled bacterium to cause disease depends not only upon the virulence factors it possesses, but also on its ability to withstand damage whilst in the aerosol state.

The inoculation of mice with aerosols of *K. pneumoniae* was chosen as a model for lower respiratory tract diseases caused by Gram-negative bacteria. This bacterium was favoured because, unlike some Gram-negative bacteria, for example *Pseudomonas aeruginosa*, *K. pneumoniae* does not produce exotoxins, so the progression and severity of the disease can be related primarily to bacterial numbers, rather than to tissue necrosis attributable to toxic agents. Inoculating mice with aerosols of *K. pneumoniae* has previously been shown to lead to lower respiratory tract infections [4, 5].

From a practical standpoint, aerosol administration of bacteria allows several mice to receive a relatively uniform inoculum. The Collison nebulizer produces aerosol particles of between about 1 and 5 μm diameter [6], and since particles of this diameter are deposited mainly in the alveoli [7], the disease will be localized in this area. Furthermore, the aerosol method of inoculation is a non-invasive technique and so the use of anaesthetics, which might affect host clearance mechanisms, is not necessary.

One objective of this study was to investigate the ability of *K. pneumoniae* to survive in the aerosol state, in order to determine whether disease might be spread from host to host by aerosolized bacteria. Several strains of *K. pneumoniae* were tested for their ability to colonize mouse lungs, with a view to finding a reproducible model of lower respiratory tract disease due to *K. pneumoniae*. The influence of the dose of *K. pneumoniae* administered to mice upon the ability of the bacteria to persist and multiply in mouse lungs was also investigated.

MATERIALS AND METHODS

Mice

The mice used in these experiments were female, between 4 and 8 weeks old and of strains BALB/c or CBA/J, obtained from Harlan Olac Ltd, Bicester, England. The mice were housed separately in a containment isolator and received food and water *ad libitum*. Each mouse received a separate filtered air supply to prevent cross-contamination from diseased mice [8].

Bacteria

The strains of *K. pneumoniae* used in these experiments were obtained from various sources. Strains 1–10 were obtained from the National Collection of Type cultures, London, England. The NCTC numbers of *K. pneumoniae* strains 1–10 were 7427, 7761, 9146, 9149, 9152, 9494, 9499, 9633, 9656, and 9667 respectively. Strains 12–14 were clinical isolates, strain 15 was 8047 of the American Type Culture Collection, Maryland, USA and strain 16 was the bacterium used by Iizawa and colleagues [5] to develop chronic pulmonary infection in mice. Spores of an isolate of *Bacillus subtilis* var *niger* were used as physical tracers in survival experiments.

Suspension fluids for the inoculation of mice were prepared by one of two methods, as follows:

(*a*) Nutrient broth 'E' was inoculated and the culture incubated with shaking for 18 h at 37 °C. Following centrifugation at 5000 g the culture was resuspended in water and used as the suspension fluid for aerosolization.

(*b*) Nutrient agar plates, comprising nutrient broth 'E' and 1·2% agar (Lab M) were inoculated and incubated for 18 h at 37 °C. The surface growth was harvested by scraping with a glass rod, suspended in distilled water and used as the suspension fluid as before. This method was adopted in an attempt to preserve the capsular material surrounding cells of *K. pneumoniae*.

Spores of *B. subtilis* var. *niger* were prepared by the method of Powers [9] and vegetative cells were removed by heating aliquots of the suspension at 60 °C for 30 min immediately prior to use.

Aerosol survival of K. pneumoniae

Suspensions containing approximately equivalent concentrations of *K. pneumoniae* strain 15 and *B. subtilis* were prepared in distilled water. These suspensions were aerosolized using a triple-jet Collison nebulizer [6] as part of a mobile Henderson apparatus [10] and the aerosols stored in a 75–1 rotating drum [11]. Samples of the aerosols were taken using Porton raised impingers [12] containing 10 ml of a solution of 1 g/l bacteriological peptone (Lab M) and 8·5 g/l sodium chloride in distilled water (P/S). Aerosol samples of ages up to 32 min after aerosolization were taken; at least three replicates were performed at each of four relative humidities (RH), nominally 20, 40, 60 and 80%. Impinger samples were diluted in P/S, plated onto nutrient agar and the plates were incubated at 37 °C for 18 h. Spores of *B. subtilis* are reasonably inert and the assumption was made that their viability was unaffected by storage in the aerosol state, thus they acted as a tracer for physical losses in the apparatus. The survival of *K. pneumoniae* was expressed in terms of the ratio of colony-forming units (cfu) of *K. pneumoniae* to *B. subtilis* and percentage viability was calculated taking the aerosol sample of one second age as 100% viability of *K. pneumoniae*.

Exposure of mice to K. pneumoniae

Mice were inoculated with *K. pneumoniae* by exposure to aerosols of the bacteria. The aerosols were generated from suspensions of *K. pneumoniae* as described previously and the aerosols were maintained at a nominal RH of 60%. Aerosols were circulated through a mouse exposure chamber, as described by Gilmour and colleagues [13]. Prior to exposure to aerosols the mice were familiarized to the restraining tubes over the course of 1 week. Mice were exposed firstly to an aerosol of distilled water for 5 min, then to the bacterial aerosol for 20 min. Finally, the chamber was flushed with a water aerosol for a further 10 min. Samples of the aerosols of one second age and also at the inlet and outlet ports of the exposure chamber were taken using Porton impingers containing 10 ml of P/S.

The concentration of bacteria within the aerosols were calculated as follows:

$$\text{aerosol concentration (cfu/l air)} = B \times V/F \times S,$$

where B = concentration of bacteria in impinger fluid (cfu/ml), V = volume of impinger fluid (ml), F = flow rate of air through impinger (l/min) and S = length of sampling time (min); cfu = colony-forming units. The bacterial concentration in the mouse exposure chamber was determined from the mean aerosol concentration, which was calculated from impinger samples taken at the inlet and

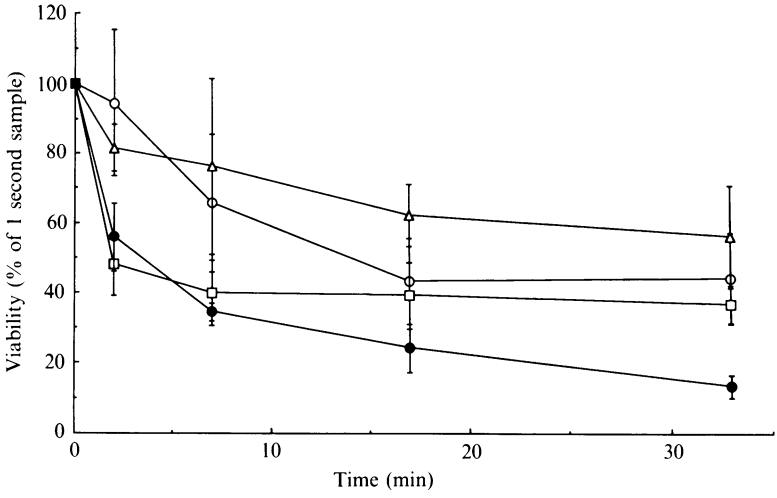

Figure 1. Viability of *K. pneumoniae* strain 15 in air. Experiments were performed at 23% (○), 40% (△), 60% (□) and 80% (●) relative humidity.

outlet ports. An estimate of the dose of *K. pneumoniae* received per mouse was calculated from the concentration of bacteria in the aerosol of the exposure chamber, using a minute volume per mouse of 20 ml [14].

Following aerosol exposure a group of between three and six mice was killed by cervical dislocation, their lungs removed and homogenized in 2 ml of P/S, then the homogenate was further diluted in P/S and plated onto nutrient agar. The plates were incubated at 37 °C for 48 h, by which time a constant colony count was obtained. Impinger fluids were treated in a similar manner to lung homogenates. Mice that were not killed immediately after aerosol exposure were returned to their cages and at timed intervals after aerosol exposure groups of at least three mice were killed, their lungs removed and homogenized and the homogenate treated as before. Mice were carefully examined at least once daily following aerosol exposure and if any mouse showed signs of sickness it was killed for humane reasons and the lungs removed for bacteriological investigation.

RESULTS

It can be seen from Figure 1 that the RH at which aerosols of *K. pneumoniae* strain 15 were stored influenced the survival of the organism. The data in this figure are expressed as mean percentage viability (± standard error [S.E.]) relative to the aerosol samples collected of one second age. The bacterium survived least well as 80% RH and best at 40% RH, however, when aerosols of 60% RH were stored there was least reduction in viability between 2 and 32 min storage. An RH of 60% was chosen for the production of aerosols to which mice were exposed, since these data suggest that aerosols of *K. pneumoniae* produced at this humidity will be most stable and so have least variability during animal exposure.

Figure 2 shows a comparison of the numbers of viable *K. pneumoniae* detected per lung in mice killed immediately after exposure with the estimated dose per lung. There is a linear relationship between the actual and estimated doses of *K.*

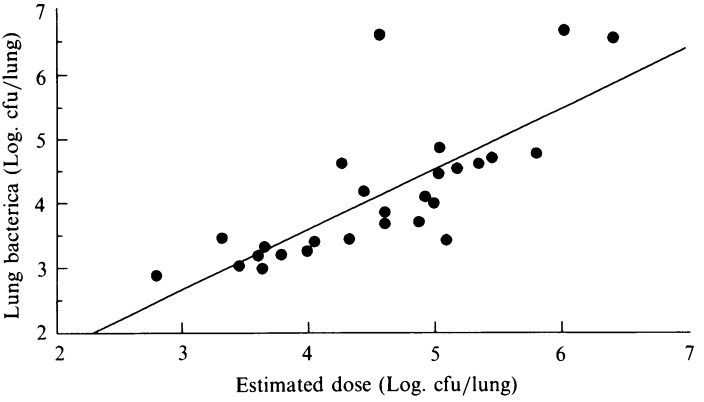

Figure 2. The relationship between actual and expected dose of *K. pneumoniae* per mouse lung.

pneumoniae per mouse lung ($r = 0{\cdot}761$) and the actual lung counts did not differ significantly from the doses estimated from the aerosols to which the mice were exposed ($P > 0{\cdot}05$, sign test).

Table 1 shows the results of exposing mice to aerosols of different strains of *K. pneumoniae*. It can be seen from the table that the majority of strains of *K. pneumoniae* tested were avirulent for mice, being cleared from mouse lungs within a few days after inoculation. Considering first *K. pneumoniae* strain 2; this strain did not cause disease in BALB/c mice, but when CBA/J mice were inoculated with the bacterium it was able to persist in mouse lungs for longer than 7 days. A similar phenomenon occurred with *K. pneumoniae* strain 3, even though the BALB/c mice received a slightly higher dose than did the CBA/J mice. Indeed, none of the BALB/c mice inoculated with aerosols of *K. pneumoniae* became colonized with the bacterium, which suggests that BALB/c are more resistant to challenge with the bacterium than are CBA/J mice. These observations support those of Iizawa and co-workers [5], who demonstrated that CBA/J mice were particularly suitable for studies requiring a consistent, persistent infection of low mortality. Thus the use of CBA/J mice alone was adopted in later experiments. In addition to *K. pneumoniae* strains 2 and 3, strain 13 also persisted in the lungs of CBA/J mice for more than 7 days. When low doses of *K. pneumoniae* strains 15 and 16 were administered to mice the bacteria persisted in mouse lungs in a similar manner to that observed with strains 2, 3 and 13. However, when the number of bacteria administered per mouse was increased, there was rapid multiplication of the bacteria within the mouse lungs.

Further studies of disease were performed with *K. pneumoniae* strains 15 and 16. Considering first strain 16, the results of exposing CBA/J mice to this bacterium are shown in Figure 3. The data points in this graph represent the mean cfu($\pm$S.E.) of *K. pneumoniae* per mouse lung. When the lower dose of approximately $1{\cdot}5 \times 10^4$ cfu/lung was used the mean numbers of viable bacteria per lung varied little over the 7-day period. However, when the initial dose per lung was raised to 4×10^4 cfu/lung the resultant disease was very different to that seen with the lower dose; a rapid multiplication of *K. pneumoniae* was observed over 48 h, so that at this time the mean lung count was 5×10^7 cfu/lung. Some of

Table 1. *The virulence of different strains of* K. pneumoniae *in mouse lungs*

Bacterial strain	Method of preparation	Mouse strain	Cfu/lung (±S.E.) at $t = 0$)	Result†
1	a	BALB/c	4·75±0·007	NV
1	a	CBA/J	4·84±0·069	NV
2	a	BALB/c	2·88±0·323	NV
2	a	BALB/c	3·39±0·133	NV
2	a	BALB/c	3·25±0·390	NV
2	a	CBA/J	3·43±0·144	P
2	b	CBA/J	4·59±0·032	NV
3	a	BALB/c	4·68±0·082	NV
3	a	CBA/J	4·44±0·144	P
4	a	BALB/c	6·50±0·019	NV
5	a	BALB/c	6·66±0·039	NV
6	a	BALB/c	6·57±0·009	NV
7	a	BALB/c	4·08±0·788	NV
8	a	BALB/c	3·84±0·049	NV
9	a	BALB/c	4·52±0·074	NV
10	a	BALB/c	3·41±0·560	NV
12	a	CBA/J	3·99±0·137	NV
13	a	CBA/J	3·69±0·089	P
14	a	CBA/J	3·66±0·034	NV
15	b	CBA/J	2·98±0·175	V
15	b	CBA/J	2·71±0·350	P
15	b	CBA/J	3·02±0·014	V
15	b	CBA/J	3·18±0·047	V
15	b	CBA/J	3·31±0·042	V
16	b	CBA/J	3·19±0·043	NV
16	b	CBA/J	4·17±0·054	P
16	b	CBA/J	4·27±0·015	V

* Suspension fluids were prepared by one of two methods (see Methods section for details).
† NV, non-virulent; P, capable of persisting in the lungs for at least 7 days without causing infection; V, virulent infection.

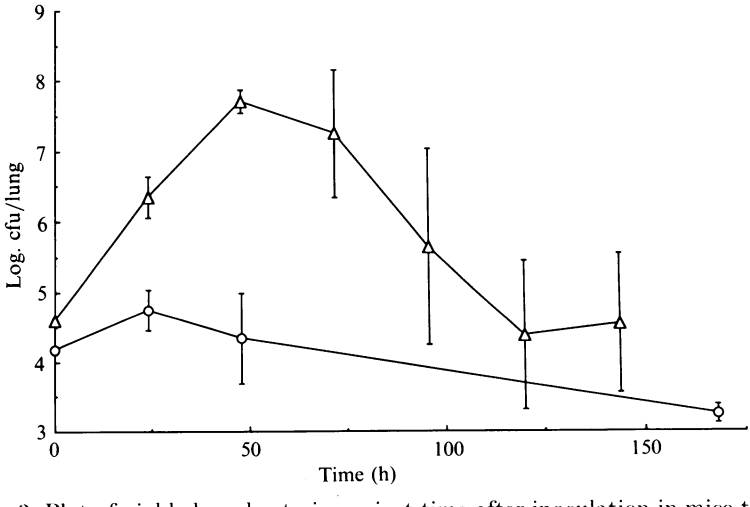

Figure 3. Plot of viable lung bacteria against time after inoculation in mice that have been exposed to *K. pneumoniae* strain 16. Initial doses used were $1·5 \times 10^4$ cfu/lung (○) and 4×10^4 cfu/lung (△).

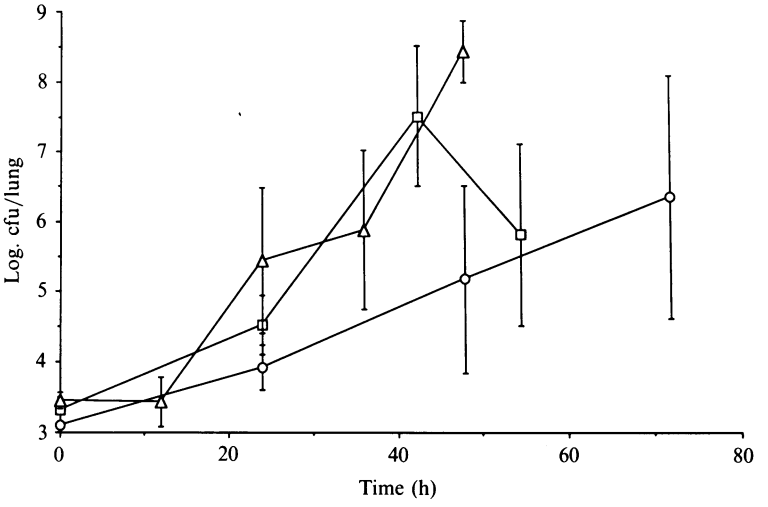

Figure 4. Plot of viable lung bacteria against time after inoculation for mice that have been exposed to *K. pneumoniae* strain 15. Initial doses used were $1{\cdot}5 \times 10^3$ cfu/lung (○), 2×10^3 cfu/lung (□) and $2{\cdot}8 \times 10^3$ cfu/lung (△).

the mice in the sample taken at this point showed signs of sickness: piloerection, hunched posture or lethargy. Four other mice, which were due to be sampled at a later time also appeared to be sick. These mice were killed for humane reasons and the lung counts are not included in the data of Figure 3, however the mean $\log_{10}$ cfu/lung of these clinically sick mice was $8{\cdot}63 \pm 0{\cdot}41$. After the 48 h sample the mean lung counts dropped progressively with time and no more sickness was observed.

Figure 4 presents the results of exposing CBA/J mice to at least 10^3 cfu of *K. pneumoniae* strain 15 per mouse lung. A net increase in the number of bacteria per lung in colonized mice was the result of all of these studies. When the lowest dose of $1{\cdot}5 \times 10^3$ cfu/lung was used, not all of the mice inoculated with the bacterium became diseased; the data represent the mean lung counts (±S.E.) of colonized mice only. However, for mice that were colonized there was a gradual increase in mean lung counts over 72 h. At this point none of the mice in the sample appeared to be sick, although clinical signs of sickness might have developed had the experiment been continued for a longer period. When the dose of bacteria received per lung was raised very slightly, to approximately 2×10^3 cfu/lung, this altered the characteristics of disease such that all mice inoculated with the bacterium became colonized. The multiplication rate was also faster than that observed when the lower dose was used; a mean lung count of above 10^7 cfu/lung was reached 42·5 h after inoculation. Those mice which received the higher dose showed slight lethargy at the 42·5 h period and this was taken to be an early sign of sickness. Sickness was more apparent 6 h later and so the final group of mice was killed at this time, though the mean lung counts were reduced compared to those sampled 6 h earlier. A more detailed study was performed, using an inoculum of almost 3×10^3 cfu/lung of *K. pneumoniae* strain 15 and sampling at 12-h intervals. It can be seen from the graph that the lung counts increased at a similar rate to that seen with the dose of 2×10^3 cfu/lung, the lung counts increased steadily up to 48 h

after inoculation, at which time sickness of mice was first noted and the final set of samples were taken. Gross lesions were observed in all lung samples taken at this time and the mean bacterial counts were $2{\cdot}5 \times 10^8$ cfu/lung of *K. pneumoniae*.

DISCUSSION

The ability of an airborne bacterium to cause respiratory disease is a function not only of its virulence, but also of its survival when in the airborne state. The survival of *K. pneumoniae* strain 15 was to some extent dependent upon relative humidity, but the cells were not particularly susceptible to the deleterious effects of storage in aerosols, since viable cells were still detectable in aerosols at all relative humidities after 32 min storage. In order to achieve the spread of respiratory disease by aerosols, microorganisms exhaled by one animal must survive until they are inhaled by another. Since this strain of *K. pneumoniae* remained viable in aerosols for some minutes, if the cells retain their ability to cause disease it is possible that disease due to *K. pneumoniae* could be transmitted by the aerosol route, even if a diseased host is not in close contact with a susceptible animal.

The numbers of viable *K. pneumoniae* detected in mouse lungs directly after inoculation did not differ significantly from the estimated dose per lung. This is in spite of several sources of uncertainty in the determination of the estimated dose of bacteria received per mouse lung. Firstly, the calculation of the concentration of *K. pneumoniae* in the aerosol of the mouse exposure chamber does not take into account the bacteria retained within the lungs of mice during exposure to the aerosols. Secondly, the number of *K. pneumoniae* within mouse lungs alone were determined, so any bacteria deposited in the upper respiratory tract would not have been detected. Some of the bacteria deposited within mouse lungs early during the exposure period would have been removed by host clearance mechanisms by the end of that period; for example Jay and colleagues [15] found that the rate at which a strain of *K. pneumoniae* was cleared from mouse lungs was 22·4% per hour. Lastly, Porton impingers sample aerosols by accelerating particles to near sonic velocity [12] and such sampling methods may be damaging to bacteria, especially those which have already been injured during aerosolization. In contrast, bacteria that are inhaled by an animal enter a warm, humid atmosphere before deposition and this may enhance bacterial survival. Thus in the absence of host clearance mechanisms the number of bacteria capable of causing lung disease may exceed the number of viable *K. pneumoniae* detected from the impingers. Considering these points, the degree of scatter observed in the relationship between actual and expected counts (Fig. 1) is not unreasonable.

When mice are exposed to aerosols of *K. pneumoniae*, serious disease can result. However, the majority of bacterial strains tested were avirulent for mice (Table 1) and of those strains that were not cleared soon after inoculation, only two were able to multiply actively within mouse lungs. The capsular materials produced by *K. pneumoniae* strains 15 and 16 were both of capsular antigen type one [5, 16] and were observed to be both more viscous and more abundant than those produced by the other strains. Ehrenworth and Baer [17] showed that strains of *K. pneumoniae* that produce large capsules were of greater pathogenicity for mice

than those strains with smaller capsules. The capsules of *K. pneumoniae* are thought to act as virulence factors by blocking the activation and binding of complement to the cell surface [18], hence phagocytosis cannot take place. Extracellular capsular polysaccharide can also inhibit macrophage maturation and function [19]. However, the capsule is not the only virulence factor of *K. pneumoniae*; Domenico and colleagues [20] found that the virulence of *K. pneumoniae* for mice is also dependent upon the amount of extracellular lipopolysaccharide produced. Straus [21] has suggested that capsule size may be the most important virulence factor of *K. pneumoniae* when the bacterium is first introduced into the lungs of an animal, but once the infection is established the release of lipopolysaccharide becomes a means of enhancing virulence.

The type of lung disease that developed in mice inoculated with *K. pneumoniae* strain 16 was dependent upon the initial inoculum administered (Fig. 3). When the lower dose was used a chronic type of disease resulted, thus there must have been multiplication of bacteria to compensate for the phagocytosis of *K. pneumoniae* by cells of the immune system. In this case the multiplication rate approximated the rate at which bacteria were cleared from the lung. Raising the initial inoculum slightly altered the characteristics of disease so that there was a rapid multiplication of *K. pneumoniae* in mouse lungs over 48 h. Toews and co-workers [22] found that mice exposed to an aerosol of 10^5 cells of *P. aeruginosa* per lung could effectively clear the bacteria from their lungs, whereas a multiplication of bacteria occurred in the lungs of mice exposed to 10^6 cfu/lung of the bacterium. They noted that the size of the inoculum had a large influence upon the ability of the bacterium to cause disease. This phenomenon might explain the results obtained with *K. pneumoniae*; if the higher dose much exceeds the number of bacteria that the alveolar macrophages can engulf, then a net increase in the numbers of viable *K. pneumoniae* will result. The decrease in lung counts that occurred after the 48 h peak could occur as a result of delayed clearance due to the presence of granulocytes within the lung environment [23].

The diseases that developed as a result of exposure to *K. pneumoniae* strain 15 were similarly dependent upon the initial dose of the bacterium. However, in this case the dose of bacteria influenced the proportion of mice to become colonized, since above an initial inoculum of 10^3 cfu/lung there was a net increase in bacterial numbers in all mice that did not clear the bacteria from their lungs. At the lowest dose of 1×10^3 cfu/lung not all of the mice that were inoculated became colonized. The most probable explanation for this is that there are slight variations between the efficiencies of the immune systems of individual mice. The slightly higher dose of 2×10^3 cfu/lung was sufficient for all mice to become colonized. This phenomenon might be explained in terms of the action of alveolar macrophages; if the dose of bacteria exceeds the concentration with which the macrophages can easily deal then bacteria may be able to multiply. The inocula used with this strain of *K. pneumoniae* were lower than those used with strain 16, yet multiplication of bacteria occurred. Thus, in spite of the similarities between *K. pneumoniae* strains 15 and 16, the former strain is substantially more virulent for the lungs of CBA/J mice than the latter.

The disease model obtained with *K. pneumoniae* strain 15 gave more consistent results and also required a lower, more easily attained inoculum than did *K.*

pneumoniae strain 16. Therefore, inoculation of CBA/J mice with *K. pneumoniae* strain 15 may be of greater use for studies requiring a reproducible model of lower respiratory tract disease involving a rapid multiplication of bacteria in the lungs.

ACKNOWLEDGEMENTS

This work was performed with the support of the Procurement Executive, Ministry of Defence.

We would like to thank Dr Y. Iizawa for the gift of *K. pneumoniae* strain 16 (identified as strain 27 in reference 5) and Mrs B. A. Handley and Miss A. Norris for their technical assistance with this work.

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